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Correspondence and
requests for materials
should be addressed to
A.C.M. (anne.moore@
ucc.ie)

Microneedle-mediated immunization of an adenovirus-based malaria vaccine enhances antigen-specific antibody immunity and reduces anti-vector responses compared to the intradermal route

John B. Carey¹, Anto Vrdoljak¹, Conor O'Mahony², Adrian V. S. Hill³, Simon J. Draper³ & Anne C. Moore^{1,4}

¹School of Pharmacy, University College Cork, Cork, Ireland, ²The Tyndall National Institute, University College Cork, Cork, Ireland, ³The Jenner Institute, University of Oxford, Oxford, UK, ⁴Dept. of Pharmacology and Therapeutics, University College Cork, Cork, Ireland.

Substantial effort has been placed in developing efficacious recombinant attenuated adenovirus-based vaccines. However induction of immunity to the vector is a significant obstacle to its repeated use. Here we demonstrate that skin-based delivery of an adenovirus-based malaria vaccine, HAdV5-PyMSP1₄₂, to mice using silicon microneedles induces equivalent or enhanced antibody responses to the encoded antigen, however it results in decreased anti-vector responses, compared to intradermal delivery. Microneedle-mediated vaccine priming and resultant induction of low anti-vector antibody titres permitted repeated use of the same adenovirus vaccine vector. This resulted in significantly increased antigen-specific antibody responses in these mice compared to ID-treated mice. Boosting with a heterologous vaccine; MVA-PyMSP1₄₂ also resulted in significantly greater antibody responses in mice primed with HAdV5-PyMSP1₄₂ using MN compared to the ID route. The highest protection against blood-stage malaria challenge was observed when a heterologous route of immunization (MN/ID) was used. Therefore, microneedle-mediated immunization has potential to both overcome some of the logistic obstacles surrounding needle-and-syringe-based immunization as well as to facilitate the repeated use of the same adenovirus vaccine thereby potentially reducing manufacturing costs of multiple vaccines. This could have important benefits in the clinical ease of use of adenovirus-based immunization strategies.

Immunization is the most successful strategy to combat infectious diseases. The creation of an effective *Plasmodium falciparum* malaria vaccine has been a much sought after goal for the vaccine community, however development of an efficacious malaria vaccine has been clinically challenging¹. Recombinant replication-defective adenoviral vectored vaccines were initially developed as candidate vaccines for induction of T cell responses against HIV-1, liver-stage malaria parasites and other intercellular pathogens². More recently, heterologous prime-boost immunization regimens, involving adenoviruses (AdV) or the poxvirus modified vaccinia virus Ankara (MVA), have shown particular promise in antibody, as well as T cell, induction in pre-clinical animal models of blood-stage malaria vaccines³. Furthermore, in mice, vaccination with an AdV-MVA regimen can protect against a lethal challenge with blood-stage and liver-stage *P. yoelii*, a significant proof-of-concept that multi-stage malaria immunity can be achieved by rational choice of antigen and of vaccine platform⁴. Promising levels of immunogenicity have been reported in proof-of-concept Phase I/II clinical trials using replication defective recombinant viral vectored vaccines based on AdV and MVA⁵⁻⁷. Therefore recombinant adenovirus-based vaccines have now become one of the leading candidate delivery platforms in the malaria vaccine field.



Table I | Microneedle Array Designs

Microneedle Array	Microneedle Height	Number of Microneedles per Array	Array Area (mm ²)	Total Pore Volume mm ³	Pore Volume Range
A	100 μ m	16	29.16	0.0018	Small
F	100 μ m	100	29.16	0.0113	Small
B	200 μ m	16	29.16	0.0145	Intermediate
D	200 μ m	25	29.16	0.0227	Intermediate
E	200 μ m	36	29.16	0.0326	Intermediate
C	300 μ m	16	29.16	0.0499	Intermediate
G	200 μ m	81	54.76	0.0734	Large
H	300 μ m	36	54.76	0.1123	Large

In the HIV field, despite acceptable safety and tolerability in early human vaccine trials, a 3000-volunteer Phase II test-of-concept study of an adenovirus human serotype 5 (HAdV5)-based vaccine was prematurely stopped due to lack of efficacy. This led to renewed focus on understanding host immunity to adenovirus vectors and how the anti-adenovirus response affects vaccine potency and efficacy. It is accepted that pre-existing humoral and cellular immunity to HAdV5 prevents repeated administration of this virus vector for booster immunizations as observed by a failure to increase immunity to the encoded antigen on repeated immunization⁸. Clinical development efforts to overcome this obstacle have focussed on the use of simian or rare human AdV serotypes with low seroprevalence in humans^{5-7,9}. However, anti-vector immunity prevents repeated use of the same serotype within a short time-frame and even heterologous AdV vectors have shown disappointing clinical results when used together in a prime-boost regimen against hepatitis C virus¹⁰. Most clinical development programmes have thus focussed on the use of AdV-MVA regimens as the leading approach for malaria vaccines³. Consequently, although new immunogenic adenovirus serotypes are being developed and are demonstrating clinical potential, the cost and logistic problem of requiring heterologous vaccines remains to be addressed. It has been demonstrated that pre-existing immunity can be overcome by vaccinating by mucosal routes¹¹⁻¹⁴, however, there has been little, if any, investigation into whether this immunogenicity can be skewed in favour of the encoded antigen (and away from the vector) by altering the method of primary immunization.

Microneedles are micron-scale needles that penetrate the stratum corneum, creating temporary conduits for drug or vaccine administration to desired depths in the skin or underlying tissue, which cannot passively permeate into the skin due to their large molecular size and hydrophilic nature. We previously demonstrated that equivalent or significantly higher immune responses can be induced by silicon microneedle patches (MN) using only approximately 3% of the vaccine dose used by the intradermal (ID) route¹⁵. Various types of microneedle systems are being developed in an effort to eliminate the need for hypodermic needles (solid or hollow microneedles) or also to stabilize vaccine out of cold chain conditions (coated or dissolvable microneedles)¹⁶. Use of a simpler vaccine delivery device that eliminates sharps waste and reduces the requirements for training in correct vaccination technique as well as in appropriate waste handling would have a positive impact on the cost-effectiveness and success of immunization programmes¹⁷.

Here, we demonstrate that, compared to ID injection with a needle and syringe, administering a recombinant HAdV5 blood-stage malaria vaccine using silicon microneedle patches decreases the anti-vector antibody response, while the humoral response to the encoded antigen is equivalent to the response induced by the ID route. The decreased anti-vector immune response induced by vaccination using silicon MN permitted successful repeated immunization with the same recombinant adenovirus vaccine. In challenge studies, the highest levels of protection against blood-stage malaria challenge were observed when a heterologous route (AdV delivered by MN

then ID) was used for priming and boosting. To our knowledge, this is the first study to demonstrate that the balance in immune responses to the virus vector compared to the encoded antigen can be favourably modulated by transcutaneous immunization.

Results

Microneedle array design does not influence the magnitude of vaccine-induced antibody responses. We previously demonstrated that the dimensions of solid silicon microneedle arrays affects vaccine-induced CD8⁺ T cell responses¹⁵. Here, we wished to initially determine if the design of the microneedle array also impacted on the induction of serum antibody responses. A range of silicon microneedle arrays were fabricated that differed in the area of the patch, the density of the microneedles, the height of each microneedle and the total pore volume of each patch. The total pore volume of the microneedle array refers to the maximum volume of the conduits that can be created in the skin after application of a microneedle array and has been arbitrarily defined as small, intermediate or large. Eight array types with increasing total pore volume that differ in microneedle density or height were fabricated (Table I).

Female C57BL/6 mice were immunized with human adenovirus serotype 5 expressing *P. yoelii* MSP1₄₂ (HAdV5-PyMSP1₄₂) by the intradermal (ID) route or using these silicon microneedle patches. The ID route was chosen as it has been repeatedly used in clinical studies and, similar to microneedles, the vaccine is delivered to skin. Serum total IgG antibody responses, examined 8 weeks after priming, demonstrated that vaccination using any microneedle patch design induced a humoral response to the 19 kDa C-terminal region of the encoded blood-stage malaria antigen (PyMSP1₁₉), that was not significantly different to ID delivery (Fig. 1A). Vaccine delivery using patch A or F resulted in a trend for lower serum antibody responses compared to other patches. Of interest, patches A and F possess the smallest total pore volume (Table I). We propose that these patch designs deliver the lowest dose of HAdV5-PyMSP1₄₂ that results in a weaker serum antibody response compared to all other forms of delivery tested here. Therefore, in contrast to CD8⁺ T cell responses¹⁵, we demonstrate that, apart from small total pore volumes (A and F), the design of the microneedle array does not impact on the magnitude of the antibody response induced by a live adenoviral vaccine. This initial study also demonstrates that microneedle patches with pore volumes in the intermediate and large range are more suitable for the delivery of antibody-inducing virus vectored vaccines.

The effect of the mode of priming on antibody responses after a heterologous boosting, with MVA, was next investigated. All groups were boosted in an identical manner, with MVA-PyMSP1₄₂ by the ID route so that post-boost immunity reflected differences in the existing primary response. A significant boost in the anti-PyMSP1₁₉ IgG response was seen in all groups, however, priming using intermediate or large pore volume microneedle arrays resulted in significantly higher post-boost serum antibody responses com-



pared to ID immunization (Fig. 1B). Interestingly, despite the weaker responses noted above, priming with small pore volume microneedle arrays (arrays A and F) resulted in equivalent post-boost antibody responses to ID priming (Fig. 1B). A significant increase in the post-boost antibody response when using microneedle-priming was unexpected, given post-prime responses were equivalent (Fig. 1A) and previous work demonstrated that adenovirus vaccine-induced antibody responses were boosted to very similar extents by a range of different vaccines¹⁸.

We also determined the PyMSP1₁₉-specific isotype profile in these immunized C57BL/6 mice. Vaccine administration using any of the microneedle arrays did not significantly change the ratio of IgG1:IgG2a eight weeks post-prime, however there was a trend towards higher IgG2a in most of the microneedle groups compared to ID immunization (Fig. 1C). A similar trend towards increased IgG2a was also observed two weeks after the MVA-PyMSP1₄₂ ID boost (Fig. 1D).

Overall, these results demonstrate that the humoral response induced by silicon microneedle arrays and ID primary immunization are quantitatively and qualitatively similar in terms of serum IgG antibody induction. However, silicon microneedle-mediated priming induces an antibody response that is significantly different with respect to responding to antigen re-exposure during secondary immunization with MVA-PyMSP1₄₂.

Microneedle-mediated immunization significantly skews the antigen-to-vector antibody response towards the antigen. We examined the effect of microneedle-mediated adenovirus delivery on the induction of anti-vector antibody responses. Surprisingly,

we determined that the antibody response to the adenovirus vector was decreased when microneedle arrays were used to administer the recombinant HAdV5-PyMSP1₄₂ vaccine compared to ID immunization (Fig. 2A). Intradermal immunization with HAdV5-PyMSP1₄₂ primed a humoral response whereby the anti-vector response was equivalent to or higher than the anti-transgene antibody response, as evidenced by an equivalent or higher relative level of vector-specific antibodies compared to antigen-specific antibodies (median ratio: 0.42; 95% CI 0.163 to 1.761). In contrast, immunization with microneedle arrays induced a response whereby the antibody response was significantly skewed by approximately 30-fold to the PyMSP1₁₉ antigen compared to the anti-vector antibody response (median ratio of all MN-treated animals: 12.90, 95% CI: 17.31 to 33.69) (Fig. 2B). This skewed response to the antigen and away from the vector also occurred when microneedle arrays A and F were used to administer the vaccine (despite lower overall immunogenicity). Finally, to determine if the kinetic of the antibody response to the transgene and to the vector was different in mice vaccinated via the ID or MN route, we examined the ratio of the anti-PyMSP1₁₉ to anti-vector response at 21, 35 or 54 days after priming (Fig. 2C). This ratio did not significantly change over 8 weeks in the ID treated group. However, in the microneedle group the ratio of the antibody response to the antigen compared to the vector steadily increased to a level where it was significantly higher compared to ID treated animals at days 35 and 54. This suggests that antibody responses develop at different rates in mice primed using micro-needle patches compared to ID administration. These results further demonstrate that there is a fundamental difference in the induction of humoral immunity to the virus vector when a recom-

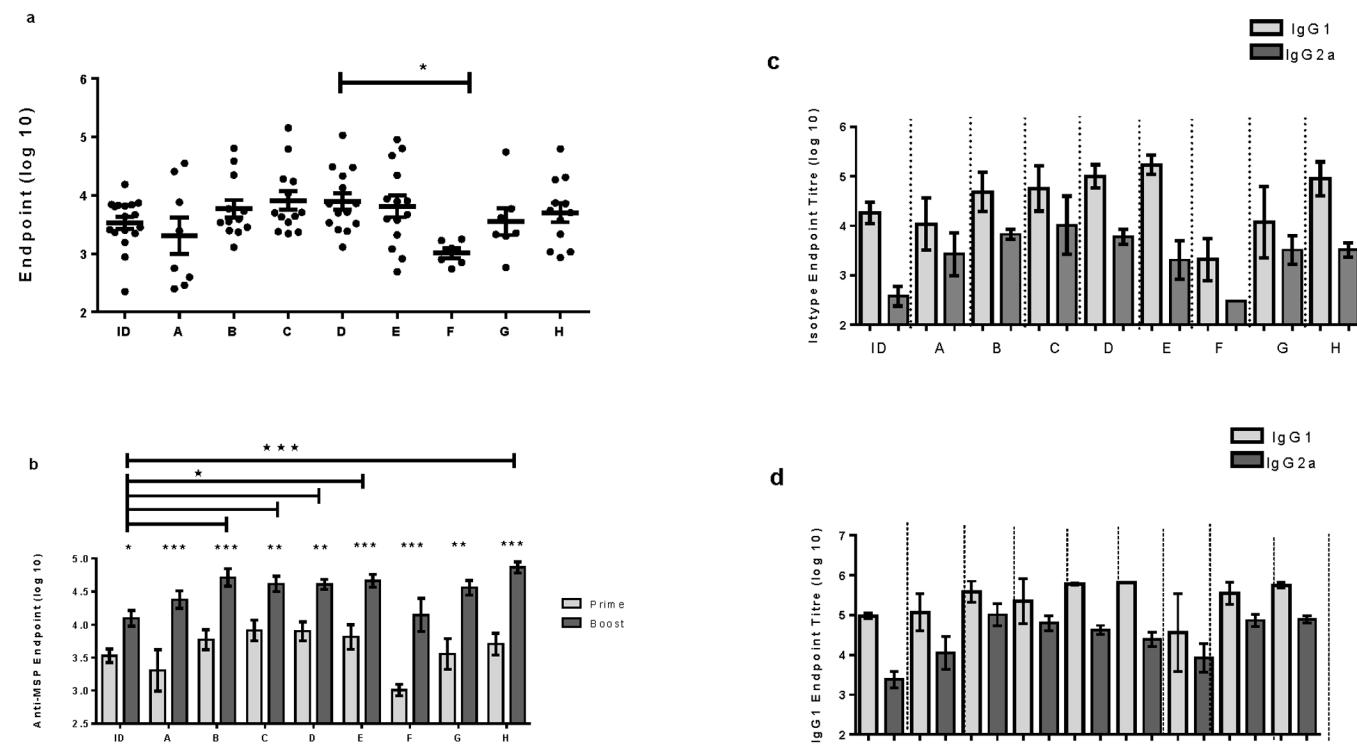


Figure 1 | Influence of the microneedle patch design on the induction of antibody responses by a recombinant adenovirus vaccine. C57BL/6 mice were immunized with 1×10^{10} vp HAdV5-PyMSP1₄₂ by the intradermal (ID) route or using a silicon microneedle patch of differing design, as outlined in Table I. All mice were boosted at 8 weeks with MVA-PyMSP1₄₂ by the ID route. Total IgG titers in blood were measured to *P. yoelii* MSP1₁₉ at 8 weeks after the primary immunization and 2 weeks after the boost. (A) Individual responses at 8 weeks post-prime. (B) Antigen-specific total IgG responses at 8 weeks post-prime (light grey bars) and 2 weeks post-boost (dark grey bars). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to post-prime antibody responses, by 2way ANOVA with Bonferroni post-test; ★ $p < 0.05$, ★★★ $p < 0.001$ compared to ID immunization by one way ANOVA using Dunn's multiple comparison test. IgG1 (light grey bars) and IgG2a (dark grey bars) antibody responses to the antigen were measured in the serum (C) 8 weeks post-prime and (D) 2 weeks after MVA-PyMSP1₄₂ boosting. The mean and standard error of the mean (SEM) ($n = 5$ to 17 mice) are shown in all panels.

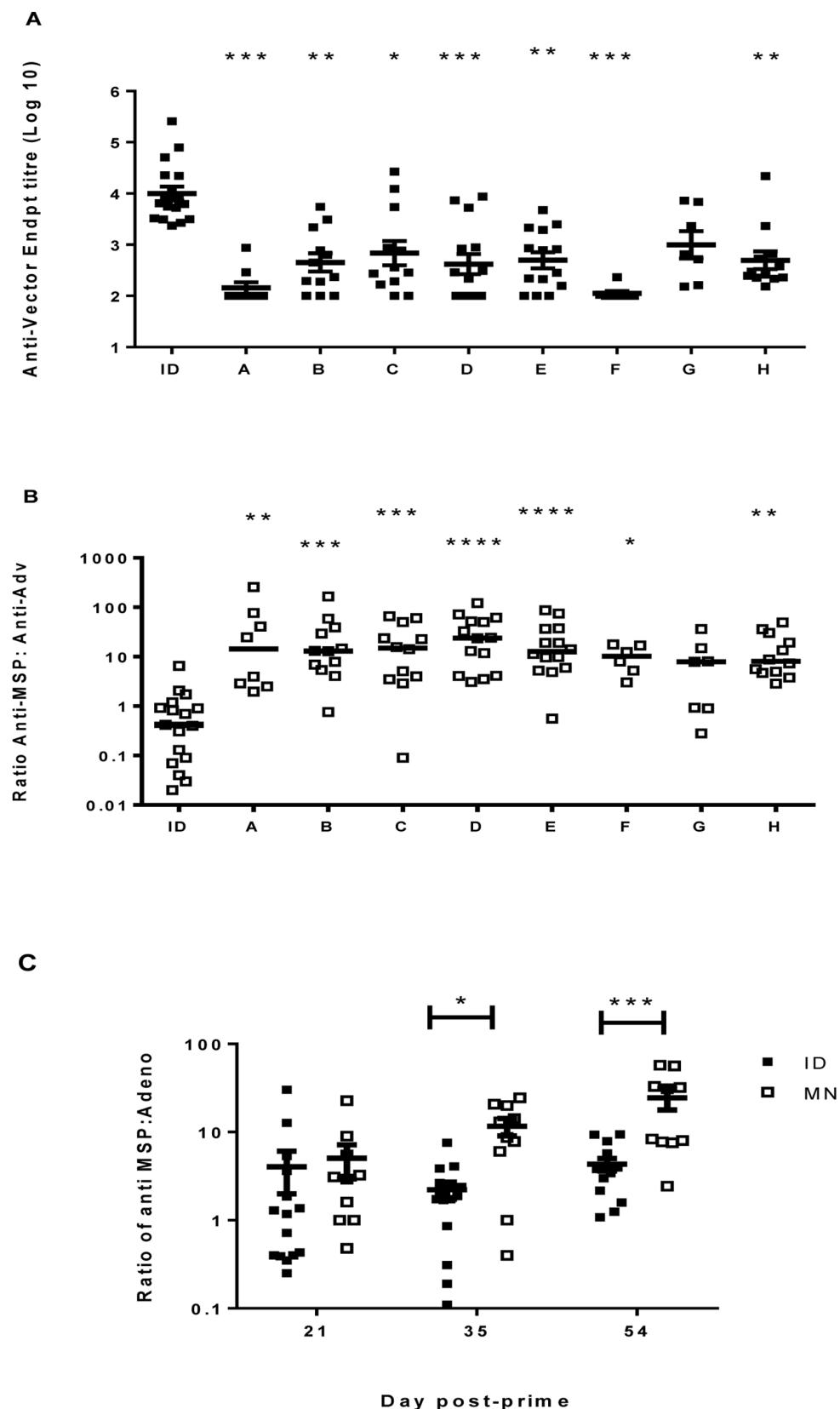


Figure 2 | Microneedle-mediated immunization alters the ratio of the antigen-to-vector antibody response. Total IgG titers to a non-recombinant HAdV5 vector (HAdV5-mCherry) in the serum of mice used in Figure 1 were measured 8 weeks after the primary immunization. (A) Individual responses at 8 weeks post-prime. (B) The ratio of antigen-specific total IgG responses to the anti-vector IgG antibody response at 8 weeks post-prime. Lines represent the median. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the ID group by one way ANOVA with Dunn's multiple comparison test. (C) The ratio of total IgG responses to the antigen and to the vector were measured at 21, 35 or 54 days after the primary immunization by the ID route or using microneedle patch type D. The mean and SEM ($n = 7$ to 15 mice) are shown. * $p < 0.05$, *** $p < 0.001$ by Mann Whitney test.



binant HAdV5-based vaccine is administered by silicon microneedle patches compared to needle-and-syringe ID delivery.

Microneedle-mediated primary immunization enhances antigen-specific immunity induced by repeated adenovirus vaccination.

Based on the finding that microneedle-mediated immunization increased the ratio of anti- PyMSP1₁₉ to anti-vector antibody responses, we next determined if primary immunization using microneedles enhanced the responses that could be induced by repeated, homologous adenovirus immunization. Mice were thus primed with HAdV5-PyMSP1₄₂ by the ID route or using microneedle patch type D. Mice were then boosted with HAdV5-PyMSP1₄₂ by the ID route, using a microneedle array on day 56 or were not boosted (“ID/–”). Serum was examined post-boost on day 74. No significant increase in anti-PyMSP1₁₉ antibody responses was observed post-boost in any animal primed by the ID route, regardless of whether the boost was performed using a microneedle patch or by the ID route (Fig. 3A). In contrast, the anti-PyMSP1₁₉ antibody response in mice that were primed with a microneedle patch was significantly increased by a HAdV5-PyMSP1₄₂ boost administered by either ID or microneedle array (Fig. 3A). Repeated immunization with HAdV5-PyMSP1₄₂ where the prime was administered with a microneedle patch and the boost administered by the ID route (MN/ID) induced the highest anti-PyMSP1₁₉ serum IgG response. With respect to anti-vector antibody induction, repeated adenovirus immunization by the ID route induced the highest anti-vector antibody titers (Fig. 3B). In contrast, the anti-vector antibody response was not significantly boosted when the delivery route was changed, i.e. ID/MN or MN/ID, to repeatedly deliver the adenovirus-based vaccine. Homologous adenovirus immunization using a microneedle array in the prime and the boost induced the lowest anti-vector antibody response. An examination of the ratio of the antibody responses to the antigen compared to the vector demonstrated that repeated delivery by ID of HAdV5-PyMSP1₄₂ immunization skewed the ratio towards the anti-vector response, compared to mice that were not boosted (Fig. 3C). In contrast, repeated vaccination using microneedle arrays induced a humoral response that was significantly skewed towards the antigen and away from the vector. These data thus demonstrate that the anti-PyMSP1₁₉ antibody response induced by recombinant adenovirus priming by the ID route is refractory to repeated adenovirus immunization, however this inability to homologously boost antigen-specific antibody response induced by recombinant adenovirus can be overcome by microneedle-mediated primary immunization.

Microneedle-mediated HAdV5-PyMSP1₄₂ immunization alters the induction of pro-inflammatory cytokines. Having observed these differences in the ability of needle-and-syringe versus microneedle delivery to prime humoral immune responses by AdV immunization, we proceeded to investigate the inflammatory profile of each platform at the site of injection and in the draining lymph nodes. We have previously demonstrated that silicon microneedle patch delivery of a recombinant MVA vaccine does not induce a pro-inflammatory response, in contrast to ID immunization¹⁵. Here, we determined if this was also true when silicon microneedle arrays were used to administer a recombinant adenovirus vaccine. We chose to examine four cytokines that have previously been demonstrated to be induced by AdV administration or have been shown to affect the induction of immunity by this vector, namely IL-1, TNF- α IL-10 and type I IFN^{19–21}. As we previously demonstrated that the analysis of cytokine proteins in skin and in lymph node homogenates was at the limit of detection¹⁵, we analyzed changes in mRNA for these cytokines at 6, 18 and 48 hours after administration of HAdV5-PyMSP1₄₂ by the ID route or using silicon microneedle array D (Fig. 4). In the skin IL-1 mRNA was elevated in both ID- and MN-treated mice at 6 and 18 hours. At the latest time point (48 h), IL-1 in the skin of ID treated mice was significantly higher compared to MN-

treated animals, which had returned to baseline. The induction of TNF- α was significantly greater in microneedle- compared to ID-treated animals at 6 h in the skin but returned to a level seen in untreated mice by 18 h. In contrast, TNF- α mRNA in the skin remained significantly greater in ID- compared to microneedle-treated animals at 18 and 48 h after HAdV5-PyMSP1₄₂ administration (Fig. 4A). For both IL-10 and type I IFN, significant increases in mRNA levels were observed in ID- but not in MN-treated mice. In the lymph nodes of microneedle-treated mice, a different response was observed compared to the skin, as no induction of these cytokine transcripts above naïve levels was observed. In contrast in the lymph nodes of ID immunized mice, at 6 h post-immunization, all cytokines were induced to a significantly higher level, compared to microneedle-treated mice. The pro-inflammatory cytokines IL-1, TNF- α , and type I IFN remained significantly higher in lymph nodes of ID- compared to microneedle-treated mice at 18 h post-immunization (Fig. 4B). Overall, these results demonstrate that, when it occurs, cytokine induction in microneedle-mediated immunization is early but transient and is only observed at the site of administration, whereas it is more sustained at the local administration site and is significantly higher in the lymph nodes of mice immunized by the ID route.

Antigen expression levels by HAdV5 differ when delivered by a microneedle patch versus ID needle-and-syringe. Having examined the inflammatory profile induced by the two vaccine delivery platforms, we also sought to assess *in vivo* antigen expression levels. Traditional delivery of AdV vaccines by needle-and-syringe has shown a very clear dose response relationship with regard to serum IgG induction against the encoded transgene, in mice²², rabbits²³ and human^{6,7} trials. Given the comparable priming of serum IgG against PyMSP1₁₉ by the HAdV5 vector administered via the ID route and most microneedle patches (Fig. 1A), we next examined the kinetics of antigen expression in ID- or microneedle-mediated delivery of a HAdV5 expressing luciferase. Substantially higher luciferase was detected at the site of immunization in ID- compared to microneedle-treated mice at day 2 and day 7 post-administration (Fig. 5A,B). The peak luciferase expression was observed 2 or 7 days after HAdV5 delivery by ID or microneedle respectively (Fig. 5B). In contrast to the sharp increase and decrease in ID-treated mice, luciferase expression was sustained at a constant level in microneedle-treated animals (Fig. 5B). Thus overall, the ID route delivers a much larger burst of antigen in a pro-inflammatory environment, in comparison to microneedles which deliver a lower quantity of transgene product under less inflammatory conditions. However, despite these differences, the priming of serum IgG against the transgene is comparable.

Repeated HAdV5 delivered by a heterologous method induces the highest vaccine efficacy against *P. yoelii* blood-stage challenge. Based on the favourable antibody responses we hypothesized that protective efficacy induced by HAdV5/HAdV5 prime/boost immunization could be enhanced if a microneedle array was used to deliver the priming vaccine. Female C57BL/6 mice were immunized using a homologous vaccine regimen (AdV/AdV); using the same or an alternate route (ID or microneedle patch) in the prime and boost. A group of mice were also immunized with the heterologous prime/boost regimen of AdV/MVA; both vaccines were given by the ID route. This group is similar to previous studies^{4,24}, except a lower dose of HAdV5-PyMSP1₄₂ and C57BL/6 mice were used. All immunized animals were challenged two weeks post-boost, at the same time as a group of naïve mice, with 10,000 red blood cells infected with lethal *P. yoelii*²⁴ (Fig. 6A). No protection was observed in the naïve animals, who were culled from day 6 to day 12. The delivery of AdV/MVA by ID/ID gave a high level of survival in C57BL/6 mice (83%), in agreement with previous studies (although slightly lower vaccine doses were used here). The use of the same vaccine and the same

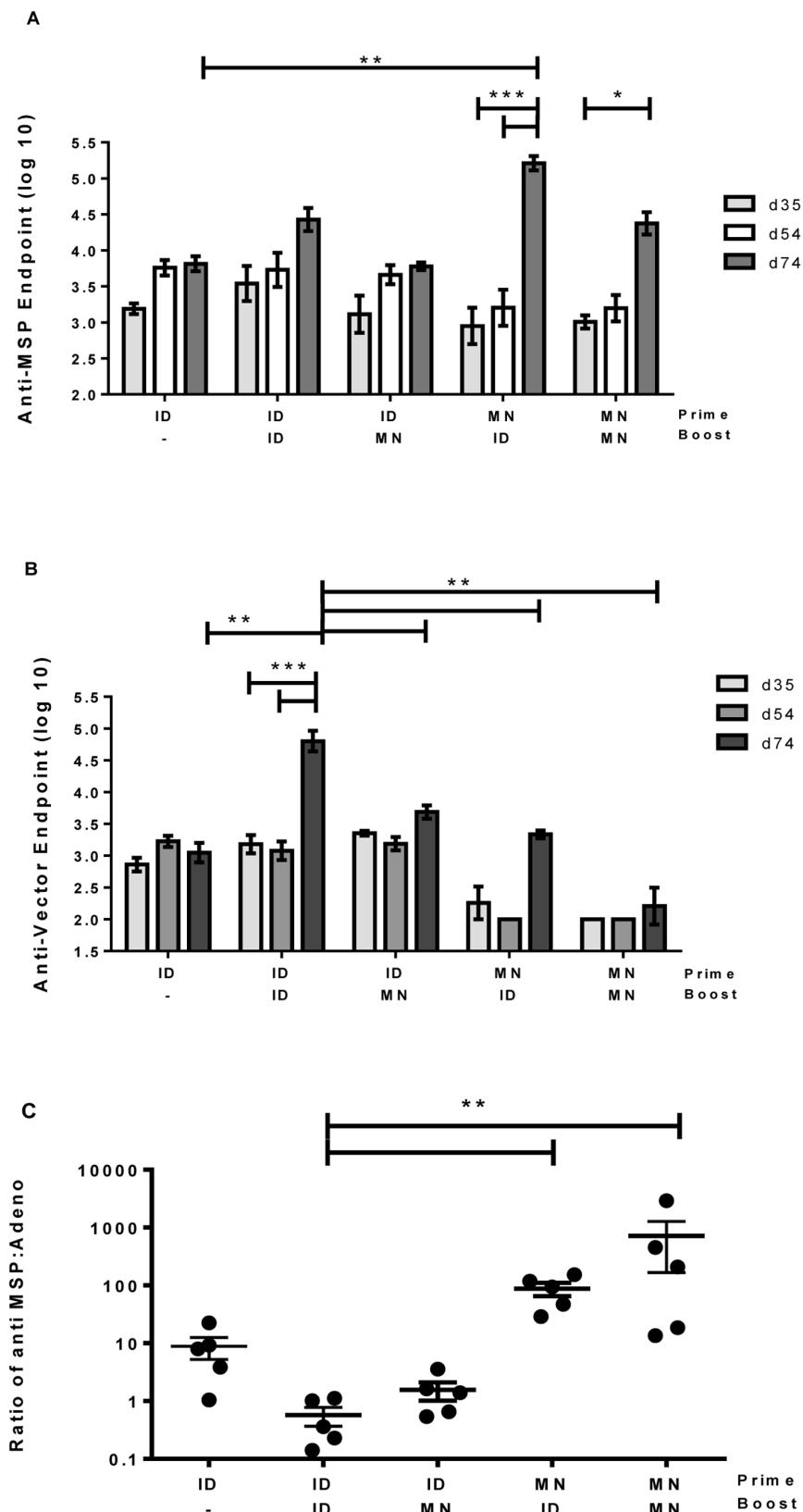


Figure 3 | Microneedle-mediated immunization permits effective boosting by the adenovirus vaccine. Mice were immunized with 1×10^{10} vp HAdV5-PyMSP1₄₂ by the intradermal (ID) route or using a silicon microneedle patch type D (MN) at day 0 and day 56, using the same (ID/ID or MN/MN) or alternative route (ID/MN, MN/ID) at each immunization. One group of mice received one immunization by the ID route (ID/–). Total IgG titers to (A) the encoded malaria antigen (PyMSP1₁₅) or (B) a non-recombinant HAdV5 vector in the serum at 35 or 54 days after the first immunization or 2 weeks post-boost at day 74. (C) The ratio of antigen-specific total IgG responses to the anti-vector antibody response at 2 weeks post-boost. The mean and SEM (n = 5 mice per group) are shown. * p < 0.05, ** p < 0.01, *** p < 0.001 by one way ANOVA with Bonferroni's multiple comparison posttest.

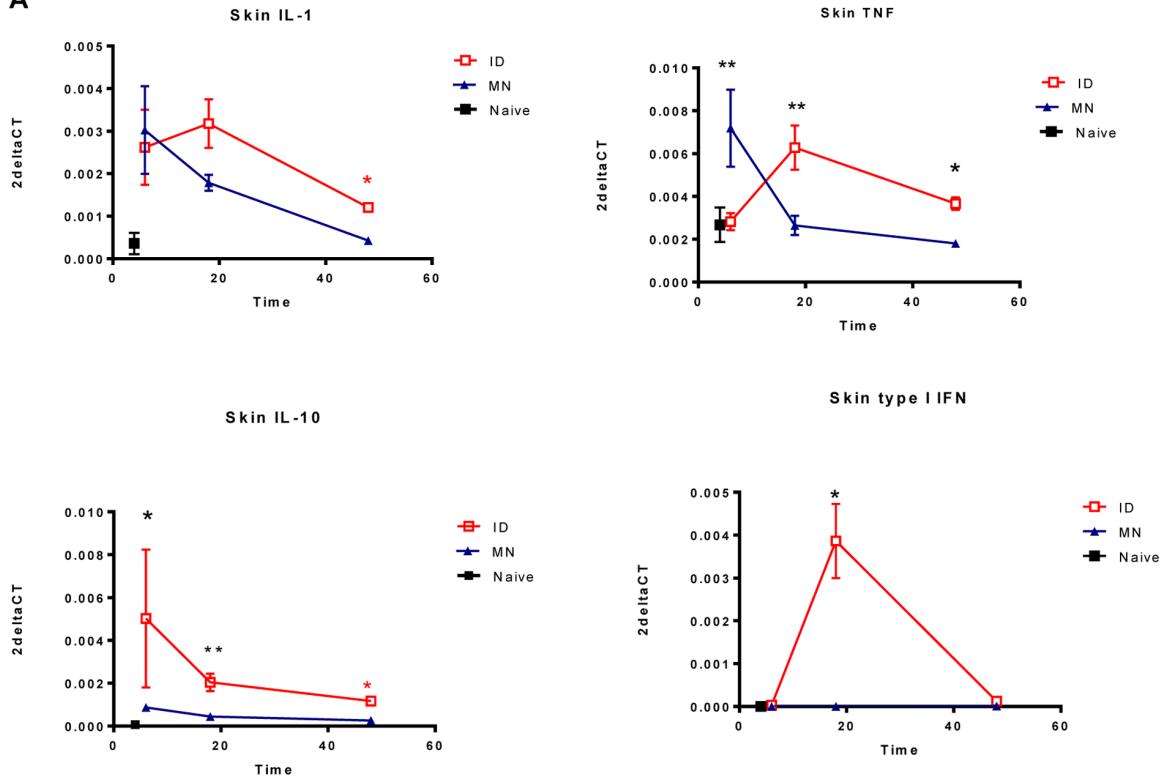
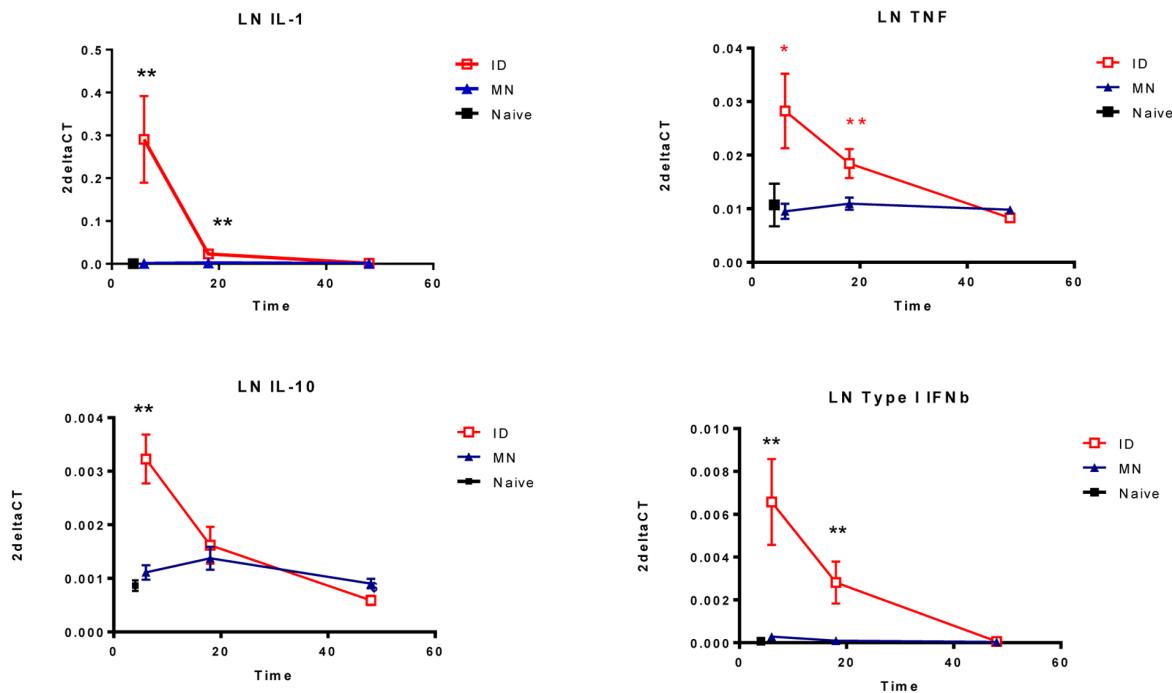
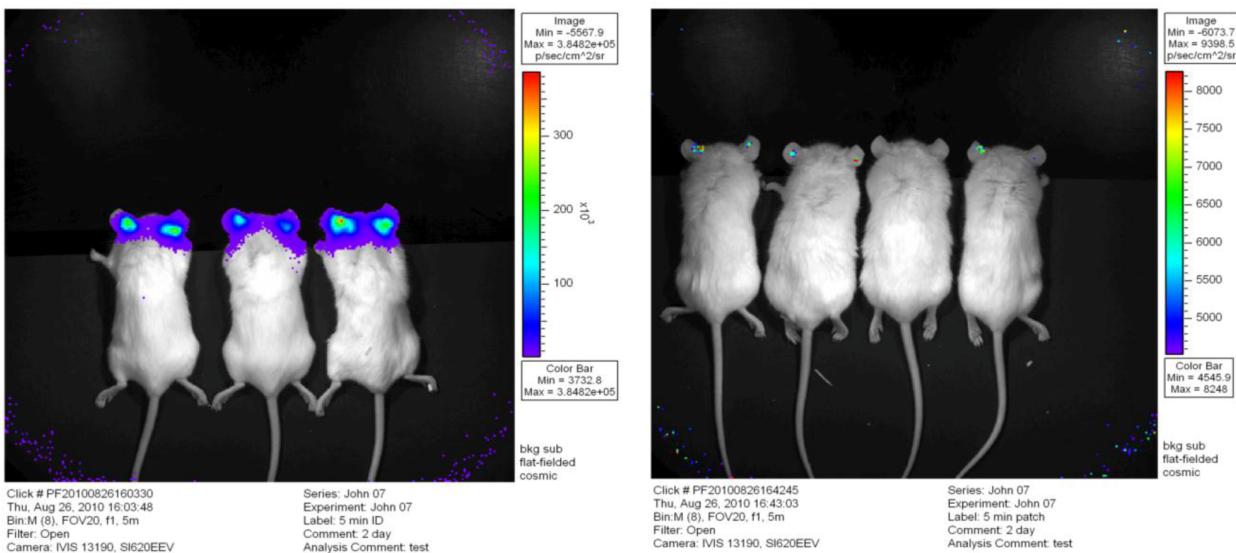
**A****B**

Figure 4 | Induction of cytokine mRNA in the skin and draining lymph node following ID or MN vaccine delivery. Mice were immunized with HAdV5-PyMSP1₄₂ by the ID route or using a silicon microneedle patch type D (MN) at time 0. At 6, 18 and 48 hours after immunization, 5 mice per vaccinated group were culled and the skin site of immunization and the draining lymph nodes were harvested and snap frozen. Skin and lymph nodes from 2 naïve mice were harvested to determine background levels of expression. Gene expression values relative to GAPDH were calculated as 2^{-ΔΔCT}. The mean (+/− SEM) values for each group are plotted; cytokine induction due to ID immunization is in red; due to MN-mediated immunization is in blue and samples from naïve mice are in black. * p < 0.05, ** p < 0.01, *** p < 0.001 by unpaired student t test of the two immunized groups.



Day 2



Day 10

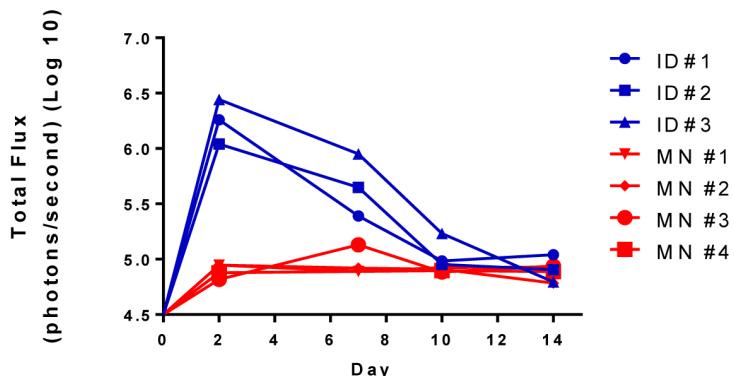
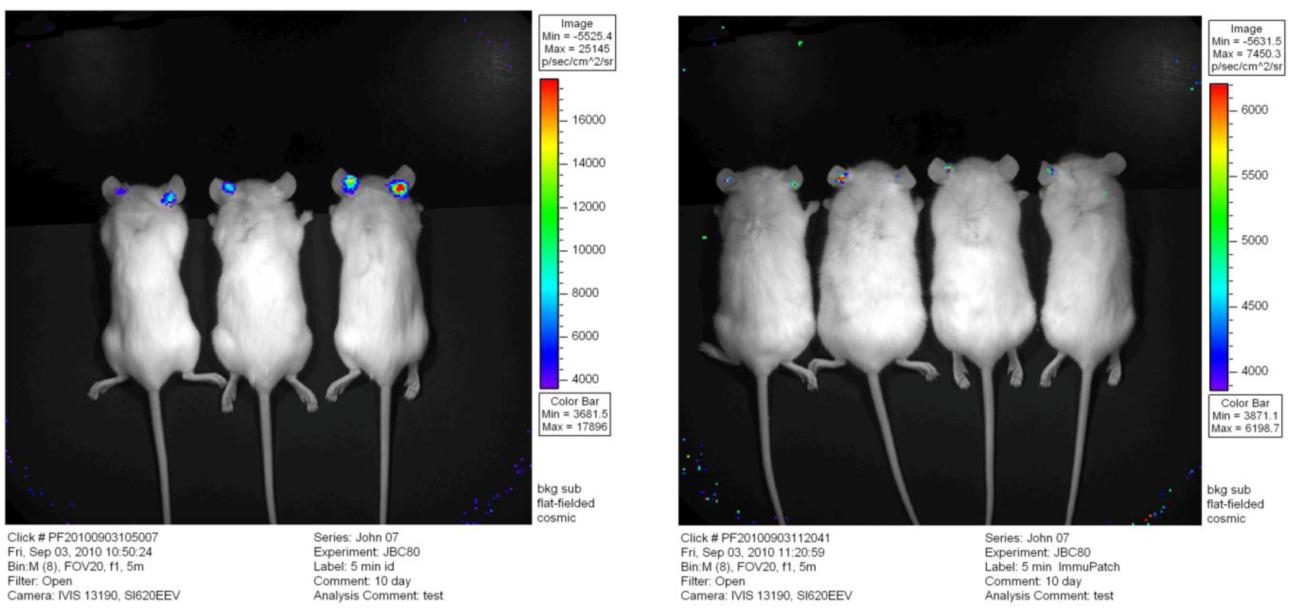


Figure 5 | Antigen expression levels by HAdV5 when delivered by ID or MN route. (A) *In vivo* imaging of luciferase expression following delivery of HAdV5 expressing luciferase to BALB/c mice by the ID (n = 3, left panels) or microneedle array type D (n = 4, right panels). Photon emissions in each group are shown at day 2 or day 10 (5 minute exposure); the emissions were measured using an RLU scale. (B) Total flux (as defined by photons emitted per second) was assessed by injection of D-luciferin at day 2, 7, 10 and 14 in each animal and is graphed on a log scale.



route in the prime and the boost (AdV/AdV MN/MN, or AdV/AdV ID/ID) induced a low level of survival (50%) with variable levels of parasitemia in surviving mice. However, administering HAdV5-PyMSP1₄₂ twice, using a microneedle patch in the prime and the ID route to boost (AdV/AdV MN/ID) induced complete survival. Five of 6 of these mice controlled the parasitemia to under 20%. The sixth animal in this group displayed a sharp increase in parasitemia on day 15 that was resolved by day 20. Therefore, complete survival and good control of parasite growth can be obtained by homologous adenovirus immunization using a heterologous regimen of microneedle-prime, ID-boost in a stringent blood-stage malaria challenge.

Discussion

Recombinant attenuated adenovirus vector-based vaccines are demonstrating strong clinical potential for malaria, hepatitis and HIV vaccines^{2,3}. However, the induction of anti-vector responses during immunization limits the repeated use of the same adenovirus vector in short time frames generally used in immunization schedules. Previous studies have examined the induction of transgene-specific immunity by mucosal routes to bypass pre-existing systemic adenovirus-specific immunity^{11–14}. In this study, we demonstrate that transcutaneous delivery of an adenovirus-based priming vaccine modulates the humoral response to one that can be boosted more effectively by re-exposure to antigen. The use of silicon microneedle transcutaneous patches to deliver the initial adenovirus-based malaria vaccine primed a humoral response that was significantly greater than intradermal-mediated immunization when animals were boosted with recombinant MVA- or adenovirus-based vaccines. Consequently, the highest levels of protection against blood-stage malaria infection was achieved in mice repeatedly immunized with HAdV5-PyMSP1₄₂ when microneedle patches were used to deliver the primary immunization and the ID route was used in the boost. These data indicate that high levels of protection can be achieved by homologous AdV immunization if utilizing a heterologous form of skin delivery.

An efficacious malaria vaccine is urgently needed to reduce the morbidity and mortality caused by this disease. However, the costs and logistics of immunization programmes must also be considered during the vaccine development process. Here we assessed if an alternative route of immunization would permit repeated immunization with the same vaccine. This would eliminate the cost of manufacturing two different vaccines required in a heterologous prime-boost vaccine regimen. Furthermore removal of hypodermic needles and syringes would have a substantial impact on the overall cost of immunization¹⁷. Thus, our objectives in this study was firstly to determine if repeated microneedle-mediated immunization would induce at least equivalent humoral immunity compared to repeated immunization using a needle and syringe by the intradermal route, and secondly to determine if repeated microneedle-mediated delivery of adenovirus in the prime and the boost was immunogenic and efficacious.

The ID route has been shown to be a highly immunogenic route for both adenovirus and MVA, and induces similar immune responses to the more clinically utilized intramuscular route^{25–27}. Although the ID route is clinically more technically difficult, it was used here as a conventional method of delivering vaccine using a needle and syringe into the same organ used by microneedle-mediated immunization. Human adenovirus serotype 5 was chosen in this study as a model adenovirus due to lack of intellectual property restrictions and its induction of comparable immunogenicity in pre-clinical models to clinically-tested simian adenoviruses^{9,28}. Our data from previous murine studies using a different transgene expressed from HAdV5 (and from human clinical trials with a related adenoviral malaria vaccine expressing the antigen METRAP⁷), demonstrated that parenteral administration of lower

doses of adenovirus-based vaccines (ID in mouse, IM in human) induced lower immune responses to the transgene and to the adenovirus vector^{26,28}. Adenovirus-naïve animals were used to model immunization of individuals that would be naïve to simian or other rare human adenovirus-based vaccines that are being developed for clinical use. We initially determined if the design of the microneedle patch would impact on the magnitude and isotype of the antigen-specific antibody response. In contrast to microneedle-mediated vaccine-induced CD8⁺ T cell responses¹⁵, no correlation between microneedle design and antibody response was observed. There was a trend for the smallest pore volume patches (designs A and F) to induce lower antibody responses. Our data demonstrate that, apart from these two patch designs, sufficient vaccine dose was delivered to the skin to induce antibody responses to the encoded antigen of an equivalent magnitude to that seen by immunization by the ID route. We previously demonstrated that the delivery efficiency of these silicon microneedle patches is approximately 3% of the applied vaccine¹⁵. Given that IgG induction by recombinant adenovirus vaccines is very dose responsive^{6,7,22,23}, the induction of equivalent transgene-specific antibody titers after the priming immunization by MN compared to ID immunization was a surprising finding. Furthermore, the significant increase in MN- compared to ID-treated mice subsequent to MVA-PyMSP1₄₂ boosting suggests that the priming immunization using microneedle patches likely induced a memory B cell response that led to stronger plasma cell responses and higher serum IgG post-boost.

We next aimed to determine if a transcutaneous route of immunization could be used to reduce the induction of anti-vector immunity during the primary immunization. Using *in vivo* imaging, we discovered that the total transgene expression was lower to that observed when the ID route was used (Fig. 5B). This is likely due to the lower dose of vaccine administered by microneedles compared to ID¹⁵. The kinetics of transgene expression was also different in the two groups; transgene expression peaked at a high level early after ID administration, whereas microneedle-mediated delivery resulted in a steady but low expression over the course of the study. We previously demonstrated that biodistribution is affected by the delivery method. Intradermal injection permits efficient antigen drainage into lymphatic capillaries. In contrast, silicon microneedle delivery did not result in effective drainage to lymph¹⁵. This lack of lymphatic drainage in microneedle treated animals may also underlie the lack of cytokine induction in draining lymph nodes (Fig. 4). We propose that, although a lower dose of vaccine is delivered by silicon microneedle patches, the adenovirus that is delivered predominantly remains at the site of delivery in the skin, infects cells and produces antigen over the duration of at least 2 weeks (Fig. 5), which results in the induction of an improved humoral response to the transgene, with a comparatively lower anti-vector response (due to the lower dose delivered by the microneedles). It is also known that a proportion of a delivered adenovirus dose consists of non-infectious virus particles²⁹, which can be viewed as a subunit anti-adenovirus vaccine. We propose that a significantly lower dose of non-infectious particles was delivered by silicon microneedle patches. Thus, although an adequate dose of viable adenovirus was delivered by the microneedle patches for *de novo* production of a sufficient dose of the encoded antigen, resulting in the induction of strong anti-PyMSP1₁₉ antibody responses, the 3% dose of non-infectious adenovirus particles was below a threshold required to induce strong anti-vector immune responses. In addition to the effect of the dose of non-viable virus particles, we propose that the lack of inflammatory responses and drainage of vector antigens to the lymph nodes are integral to the enhanced transgene-specific and reduced anti-vector responses induced by microneedle-mediated delivery of recombinant adenovirus vaccines. In effect, microneedle patches permit sufficient viable adenovirus infection to induce strong humoral immunity to the encoded antigen but insufficient delivery of non-viable adenovirus

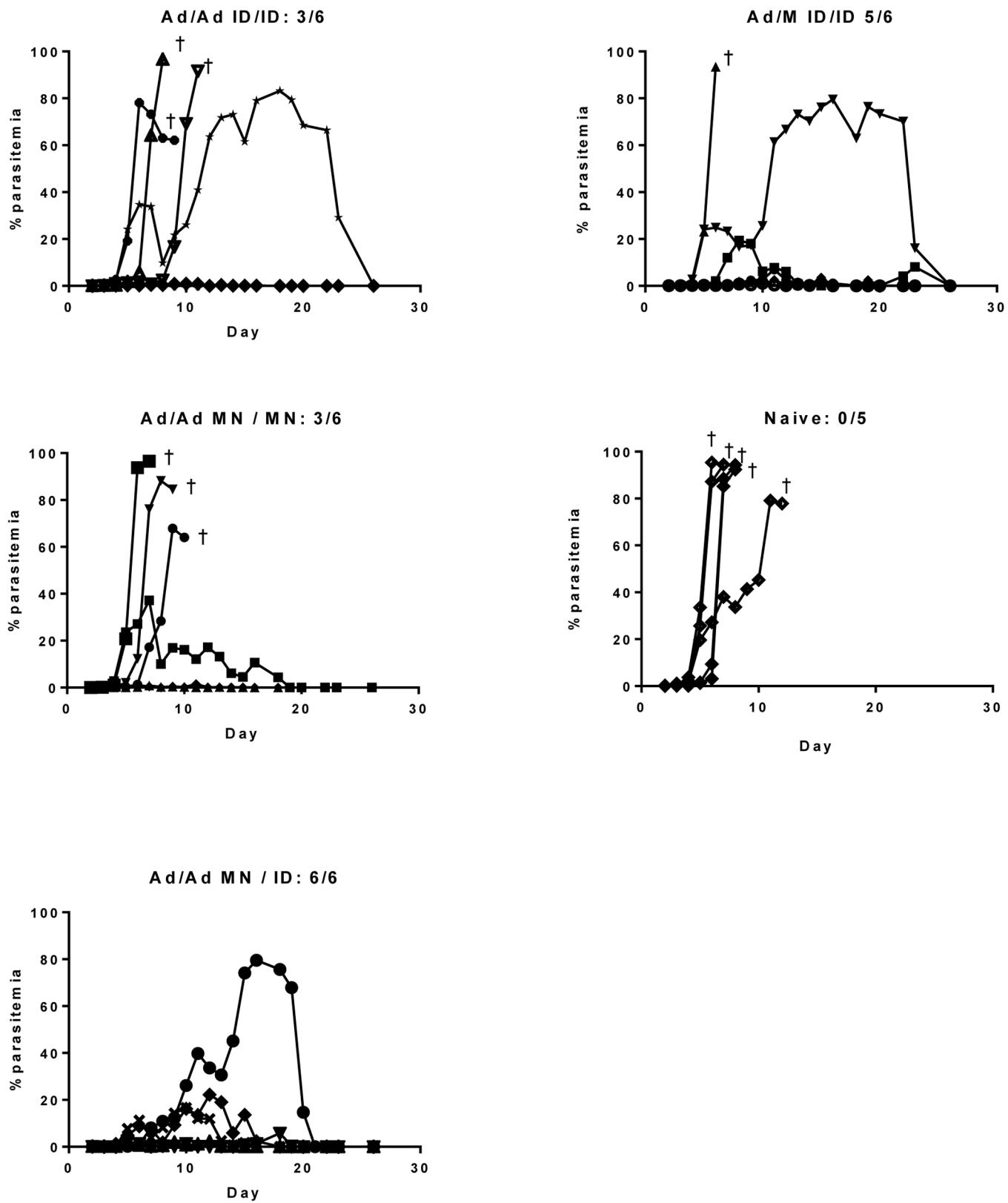


Figure 6 | The route of immunization impacts on vaccine efficacy. C57BL/6 mice were immunized twice (day 0 and 56) with 1×10^{10} vp HAdV5-PyMSP1₄₂ by the ID route or using a silicon microneedle patch type D (MN) (Ad/Ad) using the same (ID/ID, MN/MN) route in both immunizations or using a MN patch in the prime and boosting by the ID route (MN/ID). Alternatively, mice primed with HAdV5-PyMSP1₄₂ were boosted with 1×10^6 pfu MVA-PyMSP1₄₂ by the ID route (Ad/MVA). (A) Two weeks post-boost all immunized mice or naïve controls were challenged i.v. with 1×10^4 pRBCs. Parasitemia was measured as described in Materials and Methods from day 2 after challenge. The number of surviving mice, of a total of 6 mice per group, is indicated. Crosses indicate when mice were sacrificed.



particles and proteins, in the context of a non-immune stimulated environment. Other mechanisms may also be involved in driving this response, for example, different kinetics or level of induction of germinal centres, or altered induction of plasma or memory B cells. In conclusion, we propose that, in comparison to systemic needle-and-syringe ID-mediated immunization, microneedle-mediated delivery of a priming recombinant adenovirus vaccine results in more favourable induction of antibody responses to the encoded antigen and lower anti-vector immunity due to these combinations of dose, biodistribution and inflammatory factors.

We subsequently assessed the ability of a homologous HAdV5 immunization to boost these responses. In agreement with the above, effective boosting was observed when the HAdV5 was delivered by ID route – confirming the minimal impact of anti-vector responses after the microneedle prime, but suggesting ID delivery is better for AdV boosting (possibly due to dose, inflammatory environment and/or antigen expression levels). Following challenge, despite the induction of antibody responses dominated by antigen-specific responses by repeated Ad immunization with MN, repeated use of microneedle patches or of the ID route to deliver HAdV-PyMSP1₄₂ resulted in a poor survival rate – consistent with poor boosting of the transgene-specific responses and low overall antibody titers. In contrast, using a heterologous delivery regimen (MN/ID) resulted in complete survival and good control of parasitemia. This MN/ID regimen also induced the highest anti-PyMSP1₁₉ IgG antibody titers in this study, suggesting that the Ad/Ad MN/ID vaccine regimen provides protection against this stringent blood-stage malaria challenge model almost certainly by inducing high IgG antibody responses³⁰. This study therefore demonstrates that repeated adenovirus immunization can protect against blood-stage *P. yoelii* challenge when the microneedle route of immunization is used in the prime and ID is used in the boost.

In conclusion, this proof-of-concept study to determine how MN modulate humoral immunity induced by repeated adenovirus demonstrates that a heterologous route induces a higher level of efficacy against a stringent blood-stage malaria challenge in mice. Given the equivalent responses to the transgene and reduced anti-vector immunity, silicon microneedle-mediated immunization demonstrates strong potential for future development of repeatedly using the same adenovirus for immunization purposes. A limitation of this study is that it was conducted in a mouse model, which has significant differences in anatomical and physiological characteristics compared to humans. However, based on our previous studies, we have a high level of confidence that the microneedle patches used in this study penetrate human skin^{31,32}. We have also tested delivery of vaccines and drugs to *ex vivo* pig skin with these silicon micro-needles^{33,34} using accepted models of transcutaneous drug delivery; this gives us an initial level of confidence that these silicon micro-needle patches will deliver vaccine *in vivo* to humans. Furthermore, we^{6,35,36} and others^{11,37} have also previously demonstrated that immunogenicity findings in mice for adenovirus-based vaccines can translate to humans. However, in the absence of clinical testing, we cannot predict if adenovirus-based vaccine administration using solid microneedle patches would result in the same effect on immunogenicity and protection in humans as was determined in this study. Future developments where the AdV is coated onto or into MN^{38–40} would maximize the potential of this form of vaccine delivery as a feasible needle-free approach to vaccination, that aims to overcome several cost and logistic obstacles of immunization programmes in addition to enhancing vaccine efficacy.

Methods

Silicon microneedle patch design. Silicon microneedles were fabricated using wet-etch technology as previously described⁴¹. The area of each microneedle patch and the length and number of microneedles per patch were designed to produce a microneedle patch that created specific total pore volumes when inserted into skin. Each microneedle array patch was either 5.4 mm × 5.4 mm or 7.4 mm × 7.4 mm in

size. The number of microneedles per patch design ranged from 16 to 100. The pore volume of these pyramidal silicon microneedles was determined using the formula to calculate the volume of a right circular cone ($V = \pi r^2 h/3$), the effect of the length of the microneedle is therefore encompassed within the parameter of pore volume. The total pore volume per array is the sum of the volume of each pore on the array. The design and total pore volume created by each patch is detailed in Table I.

Vaccines. The construction, design and preparation of HAdV5 and MVA vaccines expressing the 42 kDa C-terminal region of *P. yoelii* strain YM merozoite surface protein 1 (PyMSP1₄₂, amino acids 1394–1757) or the fluorescent marker mCherry has been previously described^{24,28}. HAdV5 vectors were titered by UV spectroscopy as previously described to give viral particles (vp) per mL, and the MVA vector was titered to give plaque forming units (pfu). HAdV5 expressing *Renilla* luciferase was a gift from Dr. Sean Tucker (Vaxart Inc, San Francisco USA). All viruses were resuspended in endotoxin-free phosphate buffered saline (PBS) (Sigma) for immunization.

Animals and immunization. Female C57BL/6 mice, 4–6 weeks old (Harlan, UK) were used in all experiments which were conducted in strict accordance with the terms of licences from the Irish Department of Health and Children, under the Cruelty to Animals Act (licence numbers B100/4034 and B100/3157) and according to the approval of the UCC AECC Committee. Mice were immunized with 1×10^{10} vp; the same dose that was used in other studies^{8,12,14,18–20}. Vaccine was administered with a conventional 28G needle and syringe intradermally (ID) into the ear (50 μ L of 1×10^{10} vp per mouse divided across both ears). Alternatively, 5 μ L of vaccine was placed on the dorsal surface of each ear and administered to the anaesthetized mouse by pressing a microneedle array onto the ear, using a force of approximately 10–20N (1×10^{10} vp in 10 μ L per mouse). Mice were primed on day 0, and then boosted by the ID route or using a microneedle array at 8 weeks after the priming immunization using the same vaccine dose or using 1×10^6 pfu of MVA-PyMSP1₄₂.

Immunogenicity studies. Antibody responses to the vector and to the antigen were determined by ELISA as previously described^{24,39}. Briefly, antibody responses to PyMSP1₄₂ were assessed against the C-terminal moiety PyMSP1₁₉ (against which protective IgG responses are raised when using this antigen)⁴². Recombinant glutathione S-transferase (GST)-PyMSP1₁₉ fusion protein or GST control were produced as previously described^{1,24}, adsorbed overnight at room temperature (RT) to 96 well Nunc-Immuno Maxisorp plates (Fisher Scientific) at 2 μ g/mL in PBS. To detect antibody responses to the adenovirus vector, 3×10^9 vp/mL HAdV5-mCherry in PBS was adsorbed onto plates. Sera were typically diluted to 1:100, added in duplicate wells and serially diluted. Bound antibodies were detected using horseradish peroxidase-conjugated goat anti-mouse total IgG (Sigma) or biotin-conjugated rat anti-mouse IgG1 or IgG2a (BD Biosciences, Oxford, UK) followed by incubation with ExtrAvidin horseradish peroxidase conjugate (Sigma). Plates were developed by adding TNB one component substrate (Cambridge Biosciences). Optical density was read at 595 nm (OD₅₉₅). Endpoint titers were taken as the x-axis intercept of the dilution curve at an absorbance value 3× standard deviations greater than the OD₅₉₅ for naïve mouse serum (typical cut-off OD₅₉₅ for positive sera = 0.1). A standard serum sample was included in all assays as a reference control. All GST control ELISAs were negative (data not shown).

In vivo imaging studies. Delivery of replication-incompetent HAdV5 expressing luciferase was measured using an IVIS 100 charge-coupled device imaging system (Xenogen, Alameda, CA, USA). For luminescent measurements of luciferase expression, mice were imaged following intraperitoneal (i.p.) administration of D-luciferin (120 mg/kg body weight; BioThema AB, Sweden) and transferred to the imaging chamber following anaesthesia. Emission images were collected with 2, 5 or 10 min integration times. The total flux, defined by the number of photons per second, was processed using region of interest (ROI) analysis (ROIs were set to include the entire ear site) with background subtraction using Living Image 2.5 software (Caliper).

RT-PCR assessment of cytokine and chemokine induction. Ears and lymph nodes were snap-frozen in liquid nitrogen immediately after harvesting. Tissues were then disrupted using MagNA Lyser Green Beads and total RNA purified using High Pure RNA tissue kit following the manufacturer's protocol (Roche, Germany). cDNA was prepared from isolated RNA using The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Real-time quantitative RT-PCR analysis of cDNA samples, prepared from isolated RNA, for selected genes was performed using the TaqMan® Gene Expression Assays and ABI7300 Real-time PCR System instrument and software (Applied Biosystems) following the manufacturer's protocols. The relative expression of the following genes was measured: TNF- α (ID: Mm00443258_m1), IL1- β (ID: Mm00439620_m1), IL-1 α (ID: Mm00434228_m1, IL-10 (ID: Mm00439616_m1), IFN- α (ID: Mm00439552_s1). The housekeeping gene used was GAPDH (ID: 4352932E). Real-time RT-PCR data were analyzed as follows: cycle numbers at threshold crossing (C_t) values were subtracted from C_t values for a control housekeeping gene, GAPDH, to generate ΔC_t values. Gene expression values relative to GAPDH were calculated as $2^{-\Delta C_t}$.

P. yoelii pRBC challenge. Blood-stage challenge studies were performed as previously described²⁴. Briefly, C57BL/6 mice were infected with 1×10^4 red blood cells (pRBCs) parasitized with *P. yoelii* YM by the intravenous (i.v.) route. Blood-stage



parasitemia was monitored from day two post-challenge by microscopic examination of Giemsa-stained thin blood smears. Mice were deemed uninfected in the absence of patent parasitemia in 50 fields of view. Infection was considered lethal when parasitemia exceeded 80%, at which point animals were euthanized.

Statistical analysis. Data were analyzed using GraphPad Prism version 6 for Windows (GraphPad Software, San Diego, California, USA). Normality of distribution was assessed by Kolmogorov-Smirnov test. Unpaired two-tailed Student's t-test or one way ANOVA were performed, as appropriate, to compare the responses between groups.

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Author contributions

Conceived and designed the experiments: J.B.C., S.J.D. and A.C.M. Performed the experiments: J.B.C., A.V. and A.C.M. Analyzed the data: J.B.C., A.V. and A.C.M. Contributed reagents/materials/analysis tools: C.O.M. and A.V.S.H. Wrote the paper: S.J.D. and A.C.M. All authors reviewed the final version of manuscript.



Additional information

Competing financial interests: The authors declare no competing financial interests. AVSH and SJD are named inventors on patent applications covering malaria vectored vaccines and immunization regimes. JBC, AV, COM, AVSH, ACM are named inventors on patent applications covering microneedle-mediated vaccine delivery.

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